NEW PROMOTERS FOR STRAIN ENGINEERING OF *PENICILLIUM CHRYSOGENUM*

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ABSTRACT

Filamentous fungi such as *Aspergillus* and *Penicillium* are widely used as hosts for the industrial products such as proteins and secondary metabolites. Although filamentous fungi are versatile in recognizing transcriptional and translational elements present in genes from other filamentous fungal species, only few promoters have been applied and compared in performance so far in *Penicillium chrysogenum*. Therefore, a set of homologous and heterologous promoters were tested in a reporter system to obtain a set of potential different strengths. Through in vivo homologous recombination in *Saccharomyces cerevisiae*, twelve Aspergillus niger and *P. chrysogenum* promoter–reporter pathways were constructed that drive the expression of green fluorescent protein while concurrent expression of the red fluorescent protein was used as an internal standard and placed under control of the PcPAF promoter. The pathways were integrated into the genome of *P. chrysogenum* and tested using the BioLector system for fermentation. Reporter gene expression was monitored during growth and classified according to promoter strength and expression profile. A set of novel promoters was obtained that can be used to tune the expression of target genes in future strain engineering programs.

1. INTRODUCTION

Filamentous fungi fulfil an important role in industrial biotechnology because of their long history and widespread use for the production of a broad range of compounds such as antibiotics, metabolites and enzymes [238]. Typically, transcriptional and translational signals are functional across a range of filamentous fungal hosts. For example, Aspergillus nidulans was used to express the first heterologous gene encoding for the mammalian protein chymosin under control of the *A. niger* glucoamylase promoter [239]. Over the years, many promoters have been characterized in *Aspergillus* and in *Trichoderma* but relatively few examples exist for the β-lactam antibiotics producer *Penicillium chrysogenum* [271]. For instance, the constitutive promoter of the phosphoglycerate kinase gene (*pgkA*), the phosphate-repressible acid phosphatase (*phoA*) and promoters that are sensitive to carbon and nitrogen catabolite repression such as the endo-xylanese (*xylP*) and the isopenicillin-N-synthetase (*pcbC*) promoter have been used to express the β-glucuronidase (*uidA*) gene, phleomycin selection marker and penicillin biosynthesis genes [272; 273; 274; 275; 276; 277]. The *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase gene (*gpdA*) and the *A. niger* (1,4)-β-D-arabinoxylan-arabinofuranohydrolase gene (*axhA*) promoter regions [259; 278] have been used as heterologous promoters for gene expression in *Penicillium*. For further metabolic engineering and improvement of fungal production strains, a larger set of promoters of different strengths and expression profiles is needed. This requires a more systematic analysis of the performance of homologous and heterologous promoters.

An aspect in strain improvement programs is the optimization of the fermentation process for yield, speed and maximum productivity. Traditionally, microtiter plates (MTP) or shake flasks are used for high-throughput screening applications [279]. Since the monitoring of the fermentation performance typically occurs at the end of the experiment, important kinetic parameters for biomass and product formation are not measured during the screening. Because of these limitations, online monitoring systems for continuously shaken MTPs were developed [280; 281]. This BioLector system allows for the on-line monitoring of fermentation parameters like biomass formation, pH, O_2 concentration and fluorescent reporter proteins [282]. Since then, it has been widely used to characterize bacterial and yeast fermentations [283] but so far was not applied to fermentations of filamentous fungi because of the morphological complexity of these organisms [284].

Here, we have used in vivo homologous recombination in *Saccharomyces cerevisiae* to engineer promoter–reporter pathways, and expressed

these pathways in *P. chrysogenum*. The performance of the various promoters was tested during fermentations using the BioLector system.

2. MATERIALS AND METHODS

2.1. STRAINS, MEDIA, AND CULTURE CONDITIONS

Escherichia coli DH5α, restriction enzymes, DNA polymerase, and T4 DNA ligase used in this study were purchased from New England Biolabs (Beverly, MA, USA). *S. cerevisiae* CEN.PK113-7D [285] and *P. chrysogenum* DS68530 (Δ*hdf*A ΔPen-cluster) [264] were used in this study. The latter strain is derived from the industrial strain DS17690 in which the multiple penicillin gene clusters were removed [264] as well as the *hfdA* gene which encode a homolog of the Ku70 protein involved in non-homologous end-joining and the *amdS* selection marker used to deleted the β-lactam biosynthetic genes cluster [184]. All plasmids containing the promoter, open reading frame and terminator sequences used in the Golden Gate cloning system were kindly provided by DSM Sinochem Pharmaceuticals Netherlands B.V. Yeast was grown on YEP medium containing 2% glucose as described [286]. To obtain mycelium of *P. chrysogenum* for DNA isolation, fresh spores (10^8 conidiospores immobilized on 25 rice grains) were used to inoculate 25 ml of YGG medium containing in g/l: KCl, 10.0; glucose, 20.0; yeast nitrogen base (YNB), 6.66; citric acid, 1.5; K_2HPO_4, 6.0; and yeast extract, 2.0. Cultures were incubated for 24 h in a rotary incubator at 200 rpm at 25°C. For BioLector analysis, this pre-grown mycelium was inoculated in a glucose-limited defined medium for secondary metabolites production containing the following reagents in g/l: glucose, 5.0; lactose, 36; urea 4.5; Na_2SO_4, 2.9; $(NH_4)_2SO_4$, 1.1; K_2HPO_4, 4.8; KH_2PO_4, 5.2; supplemented with 10 ml of a trace element solution containing (in g/l): $FeSO_4 \cdot 7H_2O$, 24.84; $MgSO_4 \cdot 7H_2O$, 0.0125; EDTA, 31.25; $C_6H_6Na_2O_7$, 43.75; $ZnSO_4 \cdot 7H_2O$, 2.5; $CaCl_2 \cdot 2H_2O$, 1.6; $MgSO_4 \cdot H_2O$, 3.04; H_3BO_3, 0.0125; $CuSO_4 \cdot 5H_2O$, 0.625; $Na_2MoO \cdot 2H_2O$, 0.0125; $CoSO_4 \cdot 7H_2O$, 0.625. All chemicals were from Merck. Solution was adjusted to pH 6.5. The mycelium was grown in a shaking incubator at 200 rpm for 168 h at 25°C.

2.2. PROMOTER PATHWAY CONSTRUCTION

E. coli plasmid DNA of promoters, ORFs and terminators listed in Table 1 were extracted and concentrated to 75 ng/ml with double distilled water. Golden Gate cloning was performed according to the One-Pot DNA Shuffling Method Based on Type IIs Restriction Enzymes [287]. Twelve GFP expression cassettes were generated combining six *A. niger* and six *P. chrysogenum* promoters to the open reading frame of the green fluorescent protein (GFP) venus variant and to the *A. nidulans* we. The complete nucleotide sequences are shown in the Supporting Information S1. A single RFP expression cassette was made by fusion of *P. chrysogenum paf* gene promoter (Pc24g00380, antifungal protein precursor PcPAF) and *A. nidulans* AN7354.2 terminator (40S ribosomal subunit protein) to the peroxisome-targeted fluorescent protein (DsRed.SKL, termed red fluorescent protein RFP) open reading frame (Figure 1) [1; 288]. PcPAF was chosen as an internal control as it is well expressed in *P. chrysogenum* [289].

The *amdS* gene was used as selection marker for fungal transformation. The downstream region of Pc20g07090 and the upstream region of Pc20g07100 genes (named 5' IGR and 3' IGR, respectively) were used for targeted genomic integration of the promoter–reporter pathways. These regions were synthesized by PCR from the pENTRI221-amdS plasmid and from *P. chrysogenum* DS68530 genomic DNA using the oligonucleotides listed in Supplementary Information S1. *E. coli* clones with the GFP and RFP expression cassettes, the selection marker *amdS*, and the 5' IGR and 3' IGR regions, were used as PCR templates to generate DNA fragments with recombination linkers of 50 bps for the *in vivo* recombination in yeast [290]. Primer sequences necessary for construction of the cassettes (Figure 1) are listed in the Supplementary Information S2. Co-Transformation of *S. cerevisiae* CEN.PK1137D with the DNA fragments and acceptor vector pRS417 [291] was performed as described [292] using recombination-mediated PCR-directed plasmid construction *in vivo* to generate the different pathway promoter clones [293]. Plasmid DNA was isolated, amplified in *E. coli* NEB 10 beta (New England Biolabs) and analyzed by restriction analysis. Next, the plasmids were used as PCR templates for the bi-partite targeting strategy in *P. chrysogenum* [294; 295]. PCR fragments 1 and 2 were generated which each contain part of the *amdS* gene (Figure 1) (Supplementary Information S2).

The two generated fragments have a 690 bp overlap at the *amdS* gene that once recombined in the genome will form a functional *amdS* gene (Figure 1). *P chrysogenum* DS68530 was transformed with 1.5 µg of each of the bi-partite fragments. Transformants were selected on regeneration plates containing 0.1% acetamide as sole nitrogen source to select for the presence of the *amdS* gene [262].

Table 1. Promoters, reporters and terminators used to build expression cassettes.

Promoter	Associated gene	Reporter	Terminator
An02g10320	*glaA*, glucoamylase	eGFP	Anid_AN4594.2
An04g06380	*mAspAT*, mitochondrial aspartate aminotransferase		
An04g08190	Ortholog(s) ATPase activity		
An07g01960	Putative stearoyl-CoA desaturase		
An11g02040	*gndA*, 6-phosphogluconate dehydrogenase		
An16g01830	*gpd*A, glyceraldehyde-3-phosphate dehydrogenase		
Pc16g00620	*glaA*, glucoamylase		
Pc16g11100	proton-transporting ATP synthase		
Pc20g15140	strong similarity to secreted serine protease		
Pc21g21380	*pcbC*, isopenicillin N synthase		
Pc21g21390	*pcbAB*, α-aminoadipyl-cysteinyl-valine synthetase		
Pc22g16370	SHO1, osmosensor		
Pc24g00380	PcPAF, *paf*, antifungal protein	DsRed.SKL (RFP)	Anid_AN7354.2

2.3. PROMOTER PATHWAY CHROMOSOMAL ANALYSIS

For the determination of the integration of the promoter–reporter pathways into the selected intergenic region and to evaluate the gene copy numbers, genomic DNA (gDNA) was isolated after 48 h of growth in YGG medium using a modified yeast genomic DNA isolation protocol [263] in which the fungal mycelium is broken in a FastPrep FP120 system (Qbiogene, Carlsbad, CA, USA). Diagnostic primers for genomic integration site check and for gene copy number analysis of GFP, DsRed.SKL, NiaD, and γ-actin (Pc20g11630) are listed in the Supplementary Information S2. Gene copy numbers using gDNA were analyzed in duplicate with a MiniOpticon system (Bio-Rad). The SensiMix SYBR mix (Bioline, Australia) was used as a master mix for the quantitative PCR (qPCR) with 0.4 μM of primers. The following thermocycler conditions were used: 95 C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Subsequently, a melting curve was generated to determine the specificity of the qPCR reactions. The efficiency of the primers used for the copy number determination was assessed through the use of serial dilutions of gDNA. The γ-actin reference gene, *niaD*, GFP and RFP genes showed efficiencies of 98.62% (R^2 = 0.9999), 95.23% (R^2 = 0.996), 92.39% (R^2 = 0.999), 92.47% (R^2 = 0.9992), respectively.

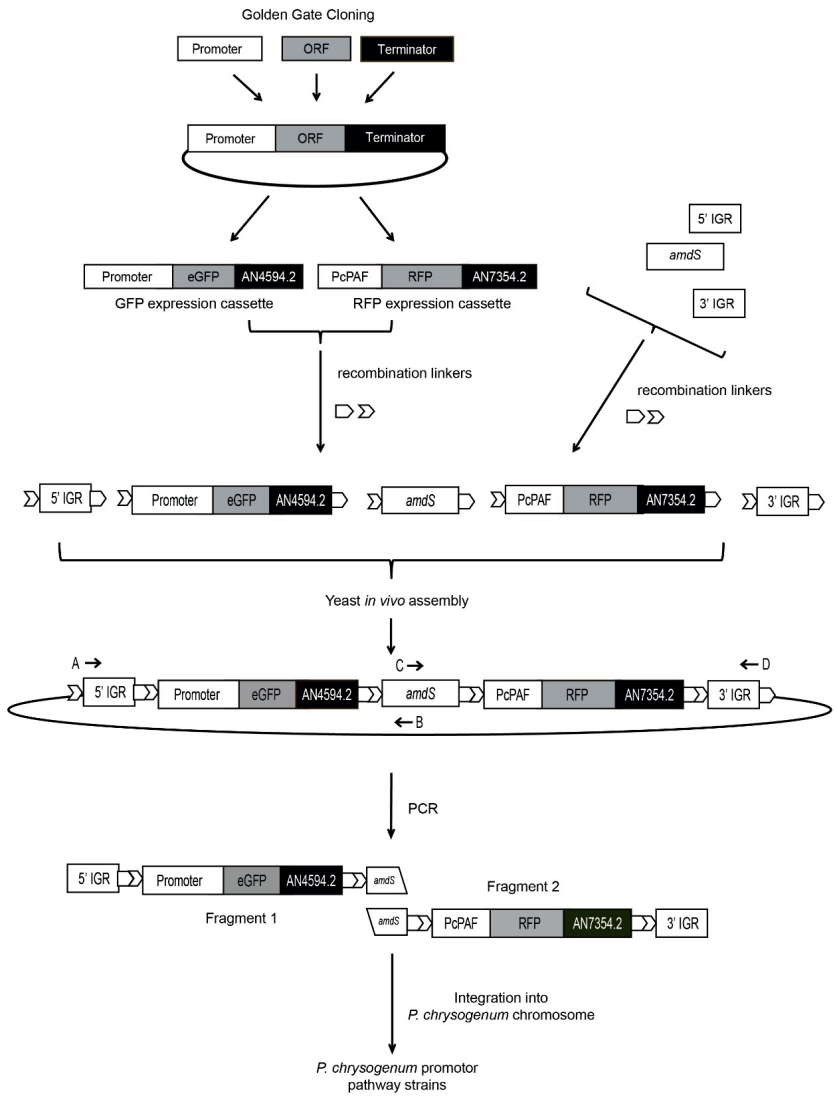

Figure 1. Promoter pathway assembling strategy. The pentagon and chevron symbols indicate the recombination linkers used for in vivo recombination in yeast. Latin letters (A–D) indicate the oligonucleotides used for the overlapping PCR to generate the fragments needed for the bi-partite integration of the promoter pathways into the *P. chrysogenum* genome.

2.4. BIOLECTOR 48 WELLS FERMENTATION WITH ONLINE MONITORING

To follow the performance of the promoter pathways, the BioLector bench top microbioreactor system (M2Plabs, Baesweiler, Germany) was used [281]. It performs high-throughput fermentations together with online monitoring of the most common fermentation parameters (biomass, pH, DO and fluorescent molecules) and runs 48 fermentations simultaneously in 1 ml wells. Pre-grown mycelium (42 h) of the different strains was diluted 8 times in 1 ml of glucose-limited defined medium to yield a cell mass of about 0.25–0.4 g/l. Cells were grown for 168 h in the BioLector at 800 rpm at 25°C. Biomass was measured via scattered light at 620 nm excitation without an emission filter. The fluorescence of GFP and RFP was measured every 30 min with 486/589 nm excitation filter and 510/610 nm emission filter, respectively. All experiments were conducted as duplicates and the mean value was calculated. In experimental repeats, different signal intensities were obtained since the sensitivity of the photomultiplier (gain) was adjusted accordingly but relative variations were similar.

3. RESULTS

3.1. PROMOTER PATHWAY ASSEMBLY STRATEGY

To obtain a set of variable promoter strengths and expression profiles to be used for gene expression in the filamentous fungus *P. chrysogenum*, information about *A. niger* and *P. chrysogenum* promoters was collected from literature and from transcriptome data [6; 264]. For instance, the promoter of the starch-regulated (*glaA*) gene from *A. niger* has been used to express several proteins like α-interferon in *A. nidulans* [243], GFP [296] or bacterial hygromycin phosphotransferase (*Hyg*) in *Ustilago maydis* [297]. Filamentous fungal promoters involved in primary and secondary metabolism were also selected. These include the *A. niger* glyceraldehyde-3-phosphate dehydrogenase gene (*gpdA*) and the isopenicillin N-synthase (*pcbC*) gene of *P. chrysogenum* [298; 299]. A list of all tested promoters is presented in Table 1. For chromosomal expression, the location of the integration site is important since it may influence gene expression [186]. Hence, the *P. chrysogenum* array data [264] was used to select an intergenic region of about 1 kb between genes Pc20g07090 and Pc20g07100 that both show medium expression levels.

To generate the promoter–reporter pathways, an approach was chosen wherein the selected promoters were used to drive the expression of GFP while the paf promoter was used to drive the expression of RFP to form an internal standard which allows for corrections in growth and biomass differences (Figure 1). The GFP and RFP expression cassettes were generated by using the Golden gate cloning technique and GFP/venus was combined with the aforementioned 12 different promoters (Biobricks of six homologous and six heterologous promoters) and the *A. nidulans* AN4594.2 terminator (Table 1). RFP, which acts as internal reference was combined with the *P. chrysogenum paf* promoter and the *A. nidulans* AN7354.2 terminator. To generate multiple overlapping DNA fragments, the amdS selection marker, GFP and RFP expression cassettes and the intergenic regions, 5' IGR and 3' IGR for chromosomal targeting, were used in a PCR reaction together with recombination linker oligonucleotides. All fragments were successfully assembled in vivo in *S. cerevisiae* into the yeast vector pRS417, and the different clones were recovered from yeast and used as template for bi-partite fragment amplification. *P. chrysogenum* DS68530 was subsequently transformed with the bi-partite fragments using the split marker (*amdS*) approach and the pathways were successfully integrated in the chromosomal site between the Pc20g07090 and Pc20g07100 genes [294]. All twelve biosynthetic promoter-reporter pathways were obtained in *P. chrysogenum* transformants and were verified for correct assembly by PCR analysis (data not shown).

3.2. GENE COPY NUMBER ANALYSIS

In order to determine whether the promoter–reporter pathway integration events were correctly targeted to the intergenic region between Pc20g07090 and Pc20g07100, genomic DNA was isolated from the new *P. chrysogenum* promoter strains and used in PCR reactions to validate the correct insertion (data not shown). However, in some cases, clonal isolates from a single transformation harboring the same promoter–reporter pathway showed large differences in the RFP fluorescence signal whereas this would not be expected for single copy transformants. To further investigate this phenomenon, isolated gDNA was used to perform quantitative PCR analysis on the GFP and RFP genes to determine their copy number, using the γ-actin and the *niaD* gene as references for single copy genes [6]. For most promoter–reporter pathways, single copy integrations were observed at the expected locus, some of the pathways showed

increased copy numbers of integration of up to two. However, the ratio of the GFP and RFP gene copy number was always one (Figure 2). This suggests that in individual cases, a double integration of the introduced pathways had occurred. However, since the GFP to RFP ratios remained the same, these transformants were further used for comparison of promoter strengths and expression profiles.

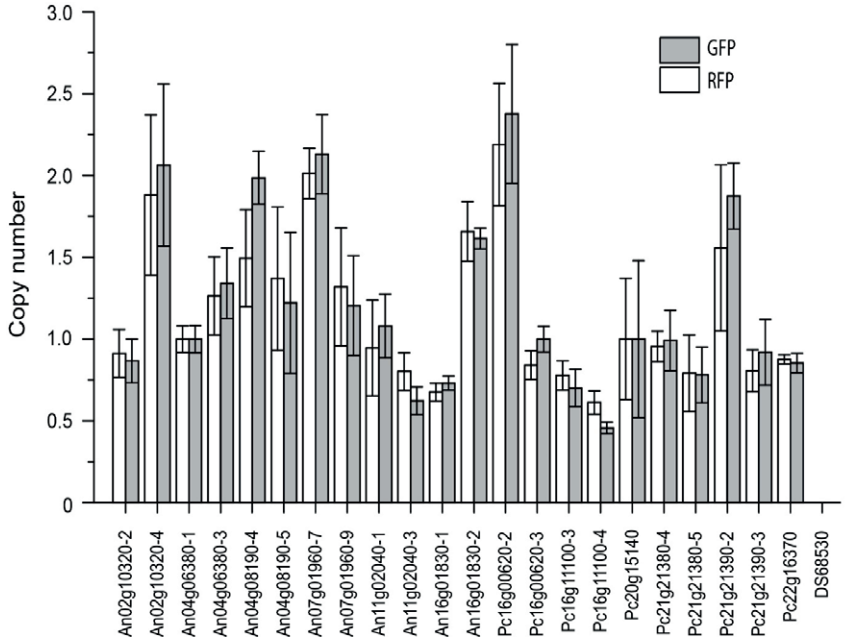

Figure 2. Quantification of the copy number of the GFP and RFP genes in *P. chrysogenum* transformants bearing a promoter–reporter pathway. Gene copy numbers vary from one to two. Strain DS68530 was used as a control that does not carry the GFP and RFP genes.

3.3. PROMOTER-REPORTER PATHWAY FERMENTATION ANALYSIS

The BioLector 48 wells fermentation system with online monitoring was used to assess the differences in promoter expression strength of each of the promoter pathways. For ten pathways, two biological replicates (individual transformants) were analyzed as well as two technical replicates each. For filamentous fungi, the mycelial biomass is either freely dispersed throughout the medium, or aggregated into clumps. Therefore the correlation between the optical density and biomass concentration is only linear during the exponential growth phase [300]. Biomass development was followed during 180 h and showed the same trend as the growth curve of a unicellular organism, but as expected differences were observed between the biological replicates due to the aforementioned filamentous fungal growth behaviour as exemplified for the intermediate *A. niger* 16g01830 (*gpdA*) (Figure 3A), and stronger *P. chrysogenum* 21g21390 (*pcbAB*) (Figure 3D).

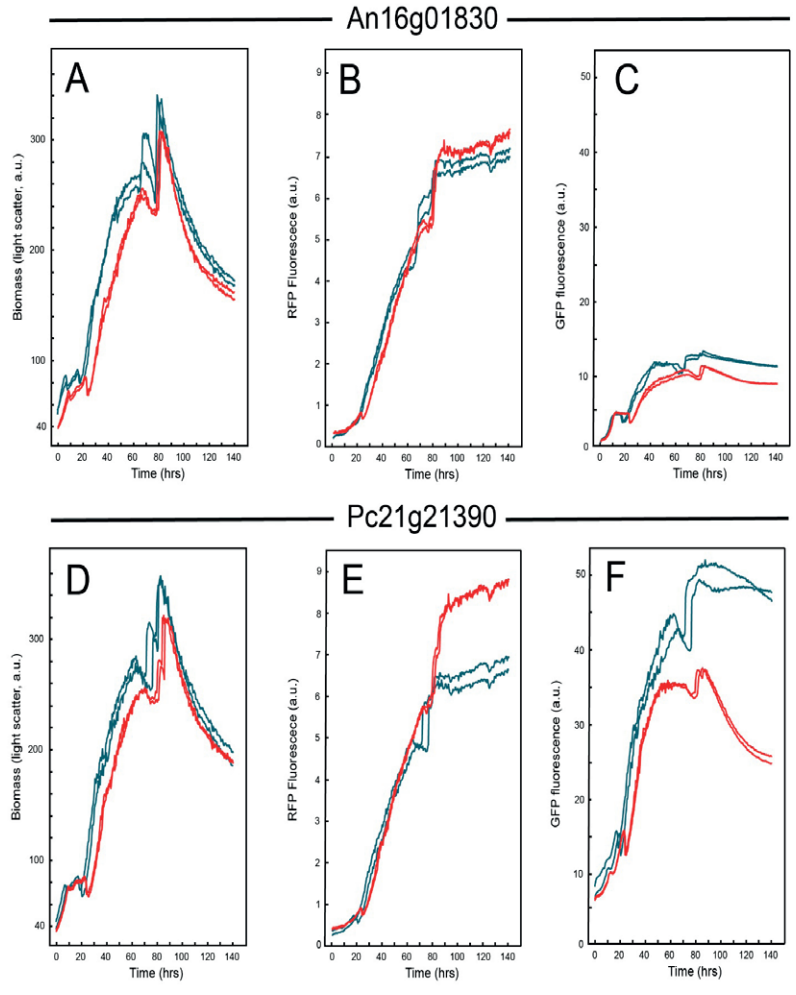

Figure 3. Development of biomass (A, D), RFP (B, E) and GFP (C, F) fluorescence in time during growth of *P. chrysogenum* harboring the promoter pathways of *A. niger* 16g01830 (*gpdA*) (A–C), and *P. chrysogenum* 21g21390 (*pcbAB*) (D–F). Data shown is for two biological replicates (individual transformants) analyzed as two technical replicates. Growth was in the BioLector system, and biomass was monitored by light scattering at 620 nm.

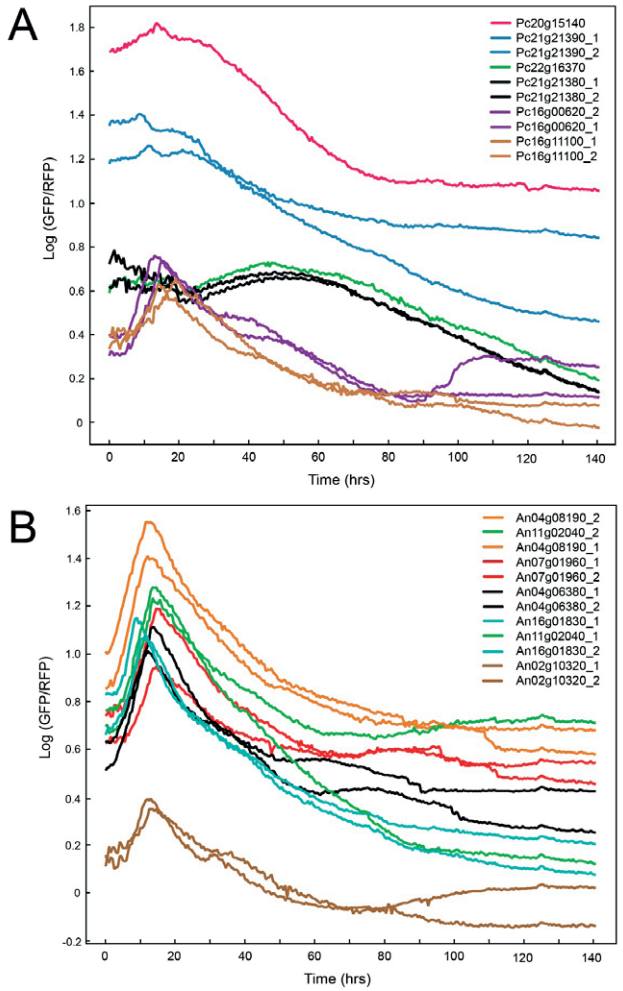

Figure 4. Activity of a range of *P. chrysogenum* (A) and *A. niger* (B) promoters in time during fermentation in the BioLector system. The promoter activity is expressed as the logarithmic values of the averaged GFP/RFP fluorescence ratios of two technical replicates. For Pc20g15140 and Pc22g16370, only one biological sample is shown.

Fluorescence was measured over a period of up to 180 h. An increases in the expression of the internal reference RFP was detected around 20–30 h, when the glucose repression on the PcPAF promoter was released, and increased linearly to 80 h when it reaches a stable plateau (Figure 3B and E). GFP gene expression showed a similar trend for the technical replicates while in some cases differences were noted between the biological replicates per promoter–reporter pathway although trends were similar (Figure 3C and F). For instance, with the promoter of the Pc21g21390 (*pcbAB*) gene that drives the expression of the α-aminoadipyl-cysteinyl-valine synthetase involved in β-lactam formation, the expression increased exponentially after the first 20 h of growth, then became more stable and in the stationary phase declined again (Figure 3F). Similar trends were observed for other transformants and this likely reflects a complex regulation of the promoters during the shift from glucose to lactose-based growth until the final depletion of sugar after about 80–100 h of fermentation. Based on these observations, we decided not to include the first and the last 40 h of analysis to catalogue the various promoters in order to have most comparable biomass development and reproducible measurements.

Using the RFP signal generated by the PcPAF promoter as a control, profiles of promoter strengths were generated for each of the tested promoters during growth (Figure 4). By taking the ratio of the GFP and RFP fluorescence, differences in growth or other variables in the analysis are eliminated. The promoter strength was analyzed in a time window of up to 180 h of fermentation. The results show that the various promoters cover a broad range of GFP/RFP ratios expressed in a log scale from 0 up to 2. There is more noise in the analysis during the first 100 h whereupon signals became more stable. All heterologous promoters were found to be functional in *P. chrysogenum*.

A box-plot graph of two smaller time windows with an average time of 40 h each was used to further catalogue the differences in the strength of the promoters (Figure 5). In this analysis, the newly investigated Pc20g15140 promoter appears to be the strongest and active in the various growth phases, while the An02g10320 promoter is the weakest. The An04g08190 promoter shows a similar strength as the highly expressing *Penicillium* promoter Pc21g21390 (*pcbAB*) [4]. The Pc22g16370 and Pc21g21380 (*pcbC*) promoters showed about 2-fold higher transcriptional activity compared to the *A. niger gpdA* promoter.

4. DISCUSSION

Many efforts have been made to improve the industrial production of peptides and proteins with antimicrobial activities [301]. One of the approaches that has been used and it showed various successes it is the production of heterologous proteins by filamentous fungi [302; 303]. In biotechnology, the main producer of β-lactam antibiotics *P. chrysogenum* is regarded as safe

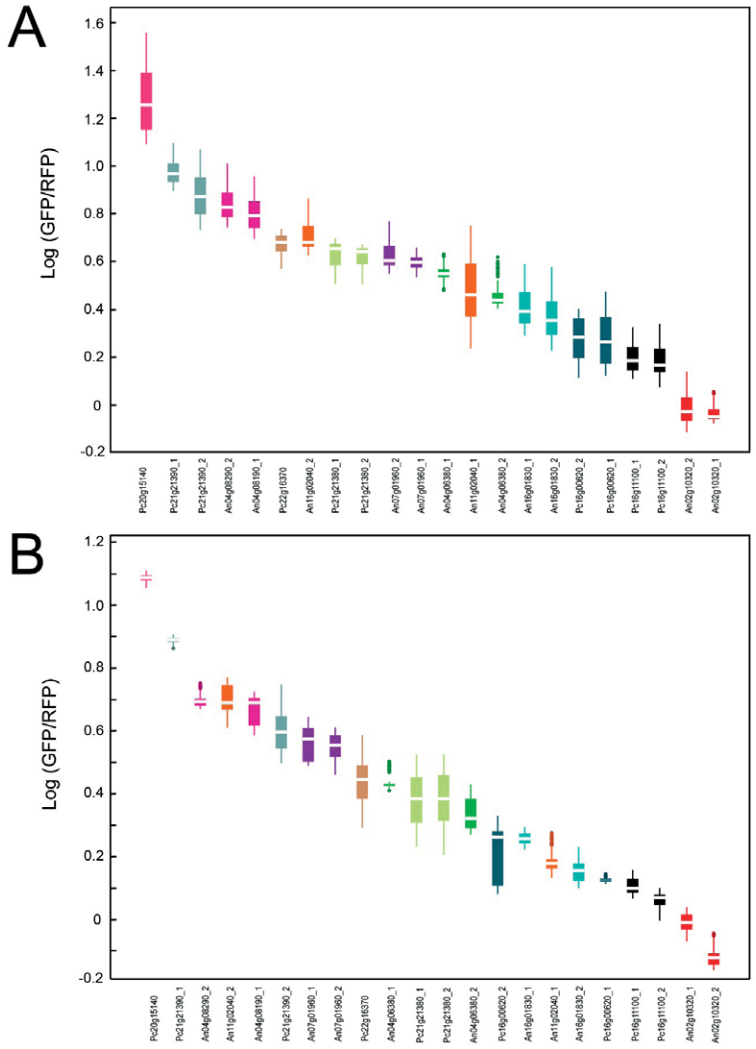

Figure 5. Ranking of the strength of 12 promoters (including biological replicates) during the time window of growth in the BioLector system from 40 to 80 h (A) and 80 to 120 h (B). The promoter activity is expressed as the logarithmic values of the averaged GFP/RFP fluorescence ratios of at least two technical replicates.

by the United States Food and Drug Administration and the existence of a well-established technology for large-scale fermentation makes this mold an interesting platform for recombinant antifungal protein production [275; 304]. However, gene expression is a multifaceted process and the factual protein production depends on many aspects in this chain such as transcription, translation, folding and degradation and often also excretion. The most controllable aspect of gene expression is transcription, i.e., the production of mRNA. One of the prerequisites to build a gene expression system is the availability of suitable promoters.

Here, we have analyzed six constitutive promoters from *A. niger*, namely An02g10320, An04g06380, An04g08190, An07g01960, and An11g02040, An16g01830 and four from *P. chrysogenum*, Pc16g00620, Pc16g11100, Pc20g15140, and Pc22g16370. For benchmarking, we added to this set, two *P. chrysogenum* promoters that drive the expression of genes involved in penicillin production, Pc21g21380 (*pcbC*) and Pc21g21390 (*pcbAB*) [305]. For each promoter, a reporter-expression system was constructed in which the GFP/venus gene was placed under control of one of the selected promoters. For comparison and internal calibration; we also integrated into the same expression cassette the gene encoding the RFP protein with the microbody targeting sequence, SKL, under control of the PcPAF promoter. This allowed us to use the GFP/RFP expression ratio as a measure of the strength of the tested promoter. By rationing, potential interferences by variations in growth are eliminated which is one of the main difficulties with growth of filamentous fungi. However, this also allowed us to deal with variable gene copy numbers. Although most constructs were integrated by single recombination events into the genomic intergenic region between Pc20g07090 and Pc20g07100 genes, a couple of constructs showed a double integration as confirmed by gene copy number analysis. This was unexpected as the strain used for transformation, *P. chrysogenum* DS68530, lacks the *hdfA* gene involved in the non-homologous end joining recombination system (NHEJ) [306] and thus only the targeted integration event should occur with high efficiency [184; 307]. It is not clear how such double integrations may have occurred. Possibly, in the Δ*hdfA* strain still some random integration occurs. Alternatively, the marker AmdS may have stimulate multicopy integration due to the way of selecting. The presence of a multicopy marker might have a growth advantage of a stronger phenotype under selection conditions.

For the internal control the red fluorescent protein RFP, a promoter of one of the most intensively studied antifungal peptides, PAF from *P. Chrysogenum* was used [289]. This protein is produced in high amounts and has severe effects on target organisms such as growth inhibition, interference with cellular metabolism and promoting oxidative stress and apoptosis [308; 309]. Significant sequence homology (42%) of PAF is detected with the antifungal protein sequence of AFP from *Aspergillus giganteus* [310]. While the *afp* gene and it expression were studied in great detail also

the expression of the *paf* gene has been studied [276; 311]. The *paf* promoter contains a TATAA box, four PACC motifs and two CCAAT consensus sequences for the binding of a HAPlike complex [312; 313]. The *paf* transcription and protein yield is maximum during the growth phase after 70–90 h of cultivation [276]. Its expression is regulated by carbon and nitrogen catabolite repression [276]. In the *paf* 5′-upstream region, four putative CREA and two GATA factor binding sites are present which might play a role in gene repression in the presence of glucose and nitrogen, respectively [314].

In this work we used conditions that are relevant for industrial production of β-lactams as a medium containing only small amounts of glucose while lactose was the key carbon source. Thus, the expression of the RFP protein occurred once the glucose was depleted from the medium. This meant that during the early stages, RFP is low expressed resulting in the peak in the GFP/RFP ratio in the first 20 h of the fermentation (Figure 4), but a stable expression signal that increased with growth was obtained during the time window 20–100 h, followed by a plateau accordingly with literature studies [311; 314]. An analogous behavior was observed for the *pcbAB* and *pcbC* promoters that are also glucose repressed. Most promoters showed a similar expression trend over time with characteristic features. The fluorescence levels increased in time consistent with the increase in biomass. However, there are two phases related to metabolic changes, i.e., the depletion of glucose followed by the consumption and exhaustion of the lactose (Figure 3). As discussed above, the depletion of glucose has a strong effect on the RFP expression and hence on the GFP/RFP ratio, but to a much lesser extent affected the expression of most tested promoters although a small bump is observed in the GFP expression. Also the depletion of lactose caused with the majority of the promoters a small decline in expression followed by a recovery phase (Figure 4). Because of these phenomena and for classification purposes, we analyzed the promoter activity in smaller time windows. The first and last windows, i.e., 0–40 and 120–160 h are less reliable because of the glucose repressing effect on the RFP expression and the death phase, respectively. Therefore, these periods were not included in the analysis. In the period of 40–120 h, a much more stable classification was obtained although small differences in the time dependent expression profile resulted in slight alterations in the ranking of the different promoters. Nevertheless, the promoter belonging to Pc20g15140, which is a secretory protein belonging to the cerato-platanin family of phytotoxins, appeared the strongest under the conditions tested within the indicated time windows, while the promoter of An02g10320 was always the weakest. These strengths covered a dynamic range of 12-fold. However, it should be emphasized that in this analysis the promoters of only well and medium expressed genes were included, while it was not the objective to reach the widest dynamic range and weak promoters were not included in the analysis. Importantly, the promoters could be benched marked against the well-known An*gpdA*, Pc*pcbAB* and Pc*pcbC* promoters.

Summarizing, we demonstrated the application of the BioLector fermentation system, for promoter strength analysis in the filamentous fungus *P. chrysogenum* and we provide a set of *Aspergillus* and *Penicillium* promoters that now can be used for developing a more versatile synthetic biology toolbox for *P. chrysogenum* and other filamentous fungi [315].

SUPPLEMENTARY INFORMATION

Table S1. Nucleotide sequences of the biobricks used in this study

Biobricks	Primer sequence (5'->3')	Short description
An02g10320	GGTCTCGGTGCTCCGGTTGTTGAGACTAGCCTGGCCAACCATATAGAGTTGAGT CATAATAACCTTGTCCGTTGTGCTTCCGAGCGGGTCTAGAAGCGGGGGAGAGGAT GAGACAAGGTTCATGATGAGGTGGTTACTGCTGGAGAACCGGAAAAGAACGCCAG GAGCACACCACTCCGGCGACAGGATCTCCAATGAGGCATCTGCTTCGTTTTCGTGGGG GTCCTGGATGTGTTTCTCGGTGAGGGAAGACGACAATCGCGGATCCTAACTTAGTA GTGGGGGTTTAGTCCAGGGTCTAGCTTGACCCCGCTGCTTCCCATTTATGCCAC GTCTTCTCCCTCCTCTCTCGCTTGCTTTCCTTTCCCTTCATCTTCCCATCTTCTCAC TATCTATCTTCAGTTATATTGTTTCCGAATAACTTACTTCTTCTTCCCCAACAACTTCCTC GTTAACCGTCCGGTACGACTCACAATGAGGCCGCGCACGCAGGATCAGACTCCG GGTCTTTTCGCTATGGGTTCAGATGGGTCAAGGTATCGTACAATAGTATAACAGT CACTGCTCGCGCATGACAGGTGTTCGGCTCGTGCTTCTGTTCCTTTCCTTCT GGTTTGGACAGGAGCGCGGTCGTTCTGAGATTATACTGTCAAAACTT GATCTAGATAATACTAGCGAAAGGACATGCGTGGCACTGATTGTCCCCTACTATTT GACCTACAGAAGACGAGAGGGATCTCGCATCCCTCTCTGTTGCTGACAGTTTC CAGACCTTTGCAATTACCCTCGACCTGAGTGTATCACCGTCAAAATGGGAGACC	Promoter used for eGFP expression
An04g06380	GGTCTCGGTGCCTACCGAGGATATCAAGAACGTAGTTAGCAGAAGGGGAATG GAGTCAATGCTGCAGATTTATATACAAGTAGTAGTTGATGGTGAGATGATGG GAAGTGGTGACTAGCAAGTGGTAGGGGGGGTGTAGATTAATTACCCCAACTCCTC GTGAGGGGAAGGCCAACCTCAGCCCATCCATGGATTTTCCCTCGATACTAAAA GAGTTCACCGGGGAGAGCGGGACGGGCTCATCATTTGTGGTGCGATCTGTCAAT GAGGGAATCCACGCTCCGTGATGATGACATTTGACATCTCATGTTAATCAGATAG TAGTCAATCAGTTAGGACTTAGTAGAGATATATACAATTCTATTCAAGATGCCATT GAATAAATAATATACTACGATGGAGTTGCATCCAAGGGATAATATGTGCAGCCT GCTTCTTGCTTCCTGCTTCCTGCTTCCTGCAGCTGCCAGCCATGCCATGCAAAC CAGCCAAACAAGCAAAAAGTCACCTGCTGGCAATGCGGAGGGCGTGGCCAATC CGATGCCTCCCGCGTTTCTCCCCGGAAACTCCCTACAGGACTAACTCGACTAGTC CAAAGGCAGTTCCAGTGACTCAAAAAATAAAATAACTATCGCCGACCTCGTCTATC CCGCTCGTCTACTCCCCCGATTCCAGCCTTCATTCAAGTACTTCTTGCCAGCTC CCTTGGCCCCGGCCTTTTCTTCTGATCATCTCCTCCCTGGTCTATTGGAGT GCTTGCTCATTTCCTCCTCTTCTTTCTTCTCTTCCCTGTTTTCCATCCCACCGT CAAAATGGGAGACC	Promoter used for eGFP expression
An04g08190	GGTCTCGGTGCAAGGGAGGGACCCGTAGAGACAAGACAAGAATGTTTTT TTCTCTCCTTTTTGTGACGACACGAGGGAAAAAAGGAATTGAACGGAAGGGATC GGTTCATACAAGTGTAAAATACACACACGACTACGGAATAATCCCATCAGATGCAG CAATGGGTTATCTGAAGGGGAAGGAGATGTGTGAGTGAATGAGAGAGTAAGC CAATGCTCCATCGCGGACCAGCACGGTCAGGTGAAGACCCTGAAACCATTGGCT GTACCAGTAGTAACTCCCCTGGTTACCCCCATCCCGAGTGATCCCGAAGGGTGTG TATGTGTGTATGTGTACACAGTATGTGTAAGGAAGTGTGGTAAGTGTGTATGTGCG GTGGAATGCCCACTGCTTTCCCGGGGGAAGGAAAAAGGATGATGAGCCAAAAAC GAGGCGCCAAACACGGTGTAAGGGAAAAAGAAGGGAAAGGATAAACTAGGGA TAACGGATGATACCAAAGACAGACACAAACAGGAAAAACAGGAACAATACAATA CAAACAAACGGTGCCAAAACACCAAACAAAAAAGTAGGTAGGGCTTTTTTTTCT GGTCCCAACAAAGCGCACTAACACCCGACGGGGGGGCTGGGTGGGAAAAGGG CAAAAAACCGCGAAAATTTAGCGGGAGAGTATTTATGTCCCGGGGGGCCTTCT GTTGTCACTTTTCCTCCAGCTTTTTCCTCCAGAAAAGTTCTCCTTCCTTCTTTC CCTTCCCAATCCCATCATTTTCTAGAGAAACTCCTCTCTCAGAACCACCACACAC CGTCAAAATGGGAGACC	Promoter used for eGFP expression

Biobricks	Primer sequence (5'->3')	Short description
An07g01960	GGTCTCGGTGCAGTGGTGCTACTCCGAGCAGGCAATACTTTGATGCGGAAAAG GAAACCGTCCCGCAATCCCAATCGGGATGGATAGCCACAGTCAAGCCACCCGAG CAATGACACCAGCCACACAGAGCGATCAAGGGGCAAAAAACGTTGGGGATTCAAC GAATGGTTGAACTGTTCTGATTGGTGGTCCGCTCCCGACCTTACCCAAAGCGG CAGCTTCTGGCCGAGCAGCGCCATCGAATCAGAGGGAGCCCAACAAGCTTAGTTG GAGGAACAGGCGGCGCTGTATGGTTGGAGATACTCCGGCCATTTGCCATCGCG GATACACTCTGCCATCCGGACACCTTCCAGACGTGCCTGGATAATACTGTGGTAG TAGTCCCCTTCCTCACGCTCCCTTTCTTGTGTCTGTTGAACCGTCGGCCACTTTGG GACCTCGGGATCTCATGATTACTTCACTGATCTACCAGTGAACTTGCCGTCAGGCCAC CCTTCTTAACTTATTCCATGCGGGTGTCCTCATAGTCGCATCATTATCATTGATTGTC CGCCTTGCTTTTCCCCAACCATCATCCGCCGGTGGACCCTGGTAGAGTTCAACT GCCTCCGAATTTTCCCTCCTTCACTGGCTCAGATCTGCCGCTTACTTCTTCGG CCTTTCGATTGCATTCCCCACCCTTTTTCTCCGTCTATTTTTTATCGCTCGCCTC CGGCTCTTATCTTTAAACCCACCCTCTCCCGCACGCATCCTTCTCCTCCTCATC CGAACCATACTGCAGGGGTCGACAATACACACGAACACCGTCAAAATGGGAGACC	Promoter used for eGFP expression
An11g02040	GGTCTCGGTGCTCTTGCGTTACGGGCGTATTTTGCTGCGGCCGGTGGTGCCCCTC CATGCCCCGCCATCTTTCAAAGCTCCTGGCGACGCCGTCATCTCCGAACATTCTC CCCCCAAAGGAATCAATTGGCAATTGGAGTCTAGTAAAGTGGTGTTTGTCATCAGTA AGGAGTTGGTGAAACTACAATCTTCCATCATGAAGAGAAGGGATATTTTTGGG GTTGTATTTTACGATGAAGGTACTGGAAATGGTGGGGGTTTTTATAGCAGTAG ACAGTCAGTCAGTAAGTAGTATGCTTGTTGTATTACCCAAACCAGATCAATC CAAAGAAAGCCTGACAGACAGCCATCAATAGATACTACTTCGTACTATAGTTAC CCACCTAACCATATTACTCAAAAAGCATCTATCTATCCGCGGGCTTCCATGCATGTC CCGGTAGCAAACTCCTCCCACCGGTGTAGTACTCTTTGGTTAGTAGTCTTGTTCACCG GAGGACTCTGCTCCTCTCCTGCTCAGGTGCTGCCCCGCCCTCCGTCCCACCATGAC GGAAGAGATGCTCCGTAAGCCGTCCAGTTGCAACGAATCCTGCTCTGACATCTTC GAACGCCTTCTCCCTTTCGCTCGCTTCTCTGCCTCTTTCCTCTCTTCCCTTTCCTTC CCCTCCAAACTAAACCTTCCTCCTTTTCTCCATCATCCTCTAGGCAGTTG GTTCTTCCTGACTGTACATATATCCACCACCTCCCCCCTCTATTCTTCCACCTCTTC CATATCTCCTTCTCCAGAGTTCATACCCCCCACACCGTCAAAATGGGAGACC	Promoter used for eGFP expression
Pc16g00620	GGTCTCGGTGCAACTCTCTGGAAATGAAGGCAGCCCCGAAGTTTTGGGATACTAG CGATCCTAGGCACTGCACCAGTCTTGAAGAGGGTCATCTCTCCGGAGATTAGTC CATCTGTGGCATTGTTTATACTTTCACACCTCCAGAACAACATGGAAGTCAAG GAATGTGGTATCAGACTCACAACCAAGAGATTTCTCACCAAAGCGCTAGTTCCAAG GCAGGTCTAGCGTGCTGACGATGGGGATAATTTAGCCGGCTAATTGGTGGACATC CGCCACCACCCCAGATTAAACGGTGGAGATGACAGGGGGCGGAGATTCAACGGGA TTAAATATCGGAGATGAAGACTCGGCATCTGCTTGAGGCAGTTAGTGCTTGATGCA ACTTGTGGTCGGTCGAAGCGATTGGCATGGTGATCAACGATCGGATAATAAGACCTCCCATGTG CCTCGGGGGATATTCGATCCGCCTGCTGAAGAGAGTAATGATGGACCTGATACTTG CAGAATCTGAACTGAAGCCCTTGACTAGCGCTGGAACTAAATTTCAAGCTAACGGT GATGCAGCAGAAGGATGACGATCTTTTCCTAACGGATTTCTCCGCAGACCCCCGAG CGCATTCTGCAATACCATGCACCTTTCATGCACCTTTCATGCAAGTTCCATG CAACTCCCACACATGTGCATTAATATGCCTTAGCTCTCTCGAATGAACTTTCACGT GGCTTAAGTCCCCTCACCTGCACCCATATAAAAGCCAAGTTCTTCCCCCACGATGA CACCAACCCCAACTCACCTTCCACCGTCAAAATGGGAGACC	Promoter used for eGFP expression
Pc16g11100	GGTCTCGGTGCACCGCGGACTGCAAATATTAATTACCCAATGGCACTCAGCG CCTGTGGTTTAATTCATTAATGTTGAAGCTTGAGGTCATAGTGTGTATAGCCCTG GAGCTACCGAGTGGAAACGATACAGGACAGGTTACAGCAAATGGACGGACAAC CGACTTGAATATATCAGCTACTCTCATAATGATGATCGAGTGCCGCACCTCTTC GCCAGGTGTTGAAAATACGTTTGTTCCATGGATGTATCAAATCAACGGAATGC CCAGCTGTATACCGCAGATACCGATGTGTTTGAGGCGGTTCTTTGCATACCTAAT CATGGGATGTTGTCAAAATAAACGCTAGTCATGTGACAGCAAGCGCTTATCAA TCTGCACCGGACTAATGTTCTTCCGCCGCATTTGTTAGGTTTAACAGCACTA AAGAAGATGGCATCGTACTCGACCAAATAATGTATGAGATACATGAGAGACTA GAGGGTTATAGTAGTTCTAATTGAATCGGATGATATAATACAAATCTAGGATCT CAACCCTTTTAAGTACGGAAACCTTGGACACAAGCGCCGTAGCCGCTTATCGATC TATATCGGACTAACGTTCTTCCACCAGCATGCTCCTGCATCACGTGCTCTCTG GCCATCGAACCGAGCAAATCCTCACCGCCATGGTTGTCACCGCCTTCCCAAGC CAGATCTCTCCCCACGTGATCTTGTCATCCCGATCCTCCGAAGTCGAACGCTTC CAAACTCACCCGAACCGTTTTCACCCCTTCAATCGAACCATACCTCCCACATCAC CGTCAAAATGGGAGACC	Promoter used for eGFP expression

Biobricks	Primer sequence (5'->3')	Short description
Pc20g15140	GGTCTCGGTGCTACTTGAGCAACATCATACGTCAACTAATTGGCACTCT TACTTTATATCTGATATGTGGTCATTGCACTAAGTAATATAATTGTCCTCGTCTAT TCAACAAGCATGTCTCCGTGGCGCAATTGGTTAGCGCGTCTGACTGTTAATCAG GAGGTTGGAAGTTCGAGCCTTCCCGGGGACGTTTCTTTTTTTCCCTTCTTTTTTTC TACTCATTAGACAGCTACTTTGTCCTTTTCTTTTTTTCTTTTGGTTTATTGAGGT CAGCTTATTGATATAATATTACATTGTGATTCAAACTCAGACGAAAATAAAATGT GGCTATGGTTATGTCCGCTCGGAGTATTTCGATGCAACCTCGGATGCAGTTGC CCTATACCGTCGCATAGCGGGAGTCGCGCTGTTCTGTGGGTCGACCATGTAATGTA ATGCTTCTGCAGATCTCGTGGAAATGGCAGCCAAGATATACCATGTCTCAGCCTG CCTGCATGCTTCCTCGTGGACCCCACAATAGTCCTCGGCCTTATTGCACCGGTTTCT GGAGGGGTATCTATTATGGGAGTATCGGCTGACGATGGGCCTGGTATGAAGG CATCCTATTTGGGCCGTGTCACCTGTGAGTCTAAGACCTTCTTCTAAGACCTGCAA CAAACGCAGCCTGCCAGTAAAGGGAAATAGAACAATATTAGACGGAAGCCTGTT GAATGGAGATATATAAACCTCGCCGGGGAGGGGACAAAACGTATACAACTAGCAAC CAAAATATTCCACACTCTCTCAAAGTATCATCAATTCCACCGTCAAAATGGGAGACC	Promoter used for eGFP expression
Pc21g21390	GGTCTCGGTGCCTTACTGGATGGGGCCGCTGGAGCCAGTGTAAAATTAGTAAC CGTATCTCGAAGTCGGAGGGTCTTTGGTGGTCTGAGATTTCAGTCGGTCCGCAC CGTGGCATTTGCAGACGGTGCGATCAGGCCAATCGTTGATGCTCGGGCAGAG CAACACTCCCCCGCTCGAAGACTAGTAAGTACTTATCATTACCGTGCCAGAAAAC GGGGCCATAGATACCCAAGTAACACCGTCGAGTCAATCGGGCTCGTGGGCCCAG CCAAGCCACGAGAGAGTAGGCAACGTGCACTCAACGACGGCGATGTTCCAAGG TAAACCGGCACGTAGAAAATGTCCGGACCACCTTGGCTCTCGTTGCAGCGTGTT GAATCTTCAGCCACCGTAAGTCGATAGCATCCGGTTAGAGTGCAACGTGGGTCT GTCTCATTCTTCTCGGTTCTTGGCACCAGAATCGGGCGTAGTTTGCCCACTGC CAAGTCGCGGGGCCGCTTGGCTGTCCCTGTGGTGGGATTTCCCGATGCAACATG CAGATACATGTAGTCGACAGTTGACAGAGCCAATGGCATCGGATCTGCCCTAGAC CGTGCTAGACGAAAGTCTCCATCTTGTCTGCGGGCAGTGCTTCAGTCGCCCAGAT TCTCGATGGAGATTGGCCAGGTCAGCCATATATACCCTGCAATGGCAGACCAATG CAGCAGGCCCAGTATAAGGAATTCCCCTCGAGCTTGTCTGTGATTGCGTTTTTTCTA ACACTTGTTGTTGCATCCGATCCGTCCCTACCAATTATTGGTCCACCGTCAAAATGG GAGACC	Promoter used for eGFP expression
Pc22g16370	GGTCTCGGTGCATGGTATTTTGGCCGATTTAAGGTATCAAGAAGATCGCCTCTCATA ATATGGCCTATGGAATACTACCTCAGGTAGCTACCTAAGACACAAAGCGGAGGT GACTAACCGGATATTTATAGATTTCCAGATCGAGTTCATTTTCATTGTTTATGTTTA GAGAATGAGGAAAAGTAAATAGACTAATGTAAACAGTTAGAATCATTCTCCAAT TATTTATTCTATTCTCAAACCGATCAAGTCCAAGCAATTTCCATTGAAATTTCCTTA ATTTCCAAAGAGAATCGAGACAGTCGATTTCCAGGGGGCCCGGACTCCACTGCG TATCTGCCACTTTTCCTCCTTTTTATCTTTCCCTCTCTCCTCCTCTCTTTCAAACT GTCAATCTTATCCACTCTTTTATTCTTTTCTTTTTATTGATTGATTCT GTCTCTTGTTTCTTTTTCCAGCCTGGTGCTGTCTTTTTCTCTCGCAAGGAGAT TCTTTTCTTTTGACTCCCAACCTCTTACCATCCCCACGGATTGTCTCTGAAC CCACTCCGGTTTTAGGTACTGTAATCCTATTATCTTTCTATGTCTTTCTTTTATATC GCTCTATTTCATTGCCACATGCAGTCACACCTGTCGTTCCCACACTAACTAACCG CAGTCACTCCTTCCCGTCCGCCTCGTTCAGGACCCGGCAATCAACAACTCTTATATA CAAGCGCATCGCTCATCACCCATCGCCATTCCCTCTTTTTCTTCGATTATTTCATAC CAATTCCATTCCACAGCCTAACCCAATCCGCACCGTCAAAATGGGAGACC	Promoter used for eGFP expression
Pc21g21380	GGTCTCGGTGCGTCGACTACATGTATCTGCATGTTGCATCGGGAAATCCCACCA CAGGGACAGCCAAGCGGCCCCGCGACTTGGCAGTGGGCAAACTACGCCCGATTCT GGTGCCAAGAACCGAGAAGAATGAGACAGACCCACGTTGCACTCTAACCGGAT GCTATCGACTTACGGTGGCTGAAGATTCAACACGCTGCAACGAGAGCCAAGGTG GTCCGGACATTTTCTACGTGCCGGTTTACCTTGGAACATCGCCGTCGTTGAGT GCACGTTGCCTACTCTCTCGTGGCTTGGCTGGGCCCACGAGCCCGATTGACTC GACGGTGTTACTTGGGTATCTATGGCCCCGTTTTCTGGCACGGTAATGATAAG TACTTACTAGTCATCGAGCGGGGGAGTGTTGCTCTGCCCGAGCACAACGATTGG CCTGATCGCACCGTCTGCAAATGCCACGGTGCGGACCGACTGAAATCTCAGAC CACCAAAGACCCTCCGACTTCGAGATACGGTTACTAATTTTACACTGGCTCCAG CGGCCCCATCCAGTAAGCATCTGGGCTGCAAGCGTATAATGTCTCCAGGTTGTCT CAGCATAAACACCCCGCCCCCGCTCAGGCACACAGGAAGAGAGCTCAGGTC GTTTCCATTGCGTCCATACTCTTCACTCATTGTCATCTGCAGGAGAACTTCCCCT GTCCCTTTGCCAAGCCCTCTCTTCGTCGTTGTCCACGCCTTCAAGTTTTCACCAT TATTTTCACCGTCAAAATGGGAGACC	Promoter used for eGFP expression

Biobricks	Primer sequence (5'->3')	Short description
An16g01830	GGTCTCGGTGCTAAGAATGGGGAAGGCGAAGGTACCGCCTTTGGGGTCCAG CCACGCGACTCCAACATGGAGGGGCACTGGACTAACATTATTCCAGCACCGGGAT CACGGGCCGAAAGCGGCAAGGCCGCGCACTGCCCCTCTTTTTGGGTGAAAGAGCT GGCAGTAACTTAACTGTACTTTCTGGAGTGAATAATACTACTACTATGAAAGACCG CGATGGGCCGATAGTAGTAGTTACTTCCATTACATCATCTCATCCGCCCGGTTCCTC GCCTCCGCGGCAGTCTACGGGTAGGATCGTAGCAAAAACCCGGGGGATAGAC CCGTCGTCCCGAGCTGGAGTTCCGTATAACCTAGGTAGAAGGTATCAATTGAAC CCGAACAACTGGCAAAACATTCTCGAGATCGTAGGAGTGAGTACCCGGCGTGAT GGAGGGGGAGCACGCTCATTGGTCCGTACGGCAGCTGCCGAGGGGGAGCAG GAGATCCAAATATCGTGAGTCTCCTGCTTTGCCCGGTGTATGAAACCGGAAAGGACT GCTGGGGAACTGGGGAGCGGCGCAAGCCGGGAATCCCAGCTGACAATTGAC CCATCCTCATGCCGTGGCAGAGCTTGAGGTAGCTTTTGCCCCGTCTGTCTC CCCGGTGTGCGCATTCGACTGGGCGCGGCATCTGTGCCTCCTCCAGGAGCG GAGGACCCAGTAGTAAGTAGGCCTGACCTGGTCGTTGCGTCAGTCCAGAGGTTC CCTCCCCTACCCTTTTTCTACTTCCCCTCCCCCGCCGCTCAACTTTTCTTTC CCTTTTACTTTCTCTCTCTCTTCCTCTTCATCCATCCTCTCTTCATCACTTC CCTCTTCCCTTCATCCAATTCATCTTCCAAGTGACTCTTCCTCCCCATCTGTCCCTC CATCTTTCCCATCATCATCTCCCCTCCCAGCTCCTCCCCTCCTCTCATCTCCTCAC GAAGCTTGACTAACCATTACCCCGCCACATAGACACACCGTCAAAATGGGAGACC	Promoter used for eGFP expression
eGFP	GGTCTCGaatgAGCAAGGGTGAAGAACTCTTCACTGGTGTTGTTCCCATTCTTGTT GAGCTTGACGGTGATGTCAACGGCCACAAGTTCTCCGTCAGCGGTGAGGGC GAGGGTGATGCCACCTACGGCAAGTTGACTCTCAAGCTGATCTGCACCACTGG CAAGCTTCCTGTTCCTTGGCCCACCCTCGTCACCACCCTCGGATACGGTCTGCAGT GCTTCGCTCGTTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCAT GCCCGAGGGCTACGTGCAGGAGCGCACCATCTTCTTCAAGGATGACGGCAAC TACAAGACCCGTGCCGAGGTCAAGTTCGAGGGTGACACCCTCGTCAACCGCATT GAGCTGAAGGGTATCGACTTCAAGGAAGATGGCAACATCCTTGGCCACAAGCTG GAATACAACTACAACTCTCACAACGTCTACATCACCGCCGACAAGCAGAAGAACGG CATCAAGGCCAACTTCAAGATCCGCCACAACATTGAGGATGGTGGTGTGCAGCTG GCGGACCACTACCAGCAGAACACCCCCATCGGTGATGGACCTGTGTTGCTCCCCG ACAACCACTACCTGTCCTACCAGTCTGCTCTCTCCAAGGACCCCAACGAGAAGC GTGACCACATGGTCCTCCTCGAGTTCGTCACTGCTGCTGGTATCACCCACGGAATG GATGAGCTGTACAAATaaaGGAGACC	eGFP enhanced Green Fluorescent Protein (Venus) (Aequorea victoria)
Anid_ AN4594 terminator sequence	GGTCTCGtaaaAATAGTTCATATTCCACTCTGGAAGGAGGGAAATGAACTGGC GCCCGCATCAACCCTTAGCTGGGTTCATGACGGTGTGGTTGTCGATGGGCTTG CAGAAGATCTAGCAACGCTGGGTCGACTTCGATACCCGTTAAAAACAGTCATA AAAATGGAAGAGTTGCAAAGCGTATACTATATATAGCTCCTATCGCTTTCGTATTGT GACTTAACTATTGTAGAGCCTGGTAGAGAAGAGTAGAACACTTGACCGCAT TATATCTGGTATTCTACAAAGCCAGTGCACCCTCGGCTAACAGAcctcGGAGACC	Used as terminator for eGFP (*A.nidulans* terminator, possible ribosomal proteins S10a)

Biobricks	Primer sequence (5'->3')	Short description
Pc24g00380 Pc Paf.pro	GGTCTCGGTGCACTTTTTTGGTCCTGATTGAAAATGGTAGCGTGGTCTAGGA GAGGTGAAGGAAGATCTAGCACTGCTTGATAACGGGTGCAATTGTCCAGTA AAGAAAGGCGTGCCTATCGTGCGATTGAAACAGAGAGCGGATGATATGTGGCG GATCTCCCAGTACAAGGCATGTTACATCTCTCCCCTAGTCGTAATTGCAAGGAT CAAACGTTGGGTCAATGGAATTCAGAGAGCTTTTCGTACGAAGTGCGTAATGTAC GTAGCATTTTATGGTAGCATGCAAAGCACATTTTGCTGCAACCCCAATTTAATGC GGTCCTGCTCAATAATTGATCTGCACTAAGGCCTTGGCGATGGGGCCAGAAAAG GGTTGTTCAGTGGTGTGTACTCCGTAATGGTCAAGCCGATTTCGAGAATGACCG TAGTGTTCATTCATCAGTGCGATATTAAATCAGTTAGCTACTCTATCTGAAAGCTA ATAAATTTCTTTACCACTAACAATACTCTTCTCTGACTGAAAGTACCTTTTC CACTCCCCTCATACTTCATGTTTTAAGCTCAACCGTAGGAAAGCCTGTATATCT TAAAAGATTTGGATTTACTCTTCCAGCGCTTACTGTCTGCTCTTTCGGCCGAGC GAACCTTGGCAGTATGATCGGACTATGTACTTTGTTACACAAAAGGAGAAGCGGG GCTGCCACTGAGGACAACCCCTGTTCAAGGGCTAGCATCCCGCTGTAAGCCCAC CCATCCCACCTTGAAGTATGCAACTTTTGACCGCCTAGACCATGTGAGCTTAT GTTACTGAAATACTACCCGCGAATCATTTCCTAATTTGCTTTGGCTCGAATCCAC CCCAGCCCTACGTAACACAACCGGGAGCTGCCTTACAGCTTGGCTGTATCACAG TATCACATAGATACATACATAGTATAGTGCCTTTGCCTTTTCGACCTATAAGCATCCG CCATATGCTAAACCTTCTCATATACCAACATTTTGGATTTGGAGATCATTTCCTAGT GAAACAACTTTATCAAATGCAATGCAGCCATCGTCCTTTGCAGATCCGAGTGGC CCAGTCACCGTGTCAACGTGTCAGCCGTTTTCTCTGCTTTTTAGGAAATGATTAC CACTAGGTAAGCCCAAAAATATCTTCCTGGTAAACAAGTAGTGCATCTTACCCCG GAGGCTGAAGCAGGTAAGGGATTTTGGAGAGAGCCCACCCGTAAGAATATACCAG CCAAGAGGTCCAGTATCCTGAAGTATGTGAGGCATTAATGTCATTGGAGAAGTCAT GCAATCCATAAGCTGCCACCCCCAAGATGACTGCATTGGACCTGAGCATTGTATGT GTCACCTTTCACACAGAGCTCATGATCTGGTTTATAAAGGCGGCTTCATGACCCT CAATTCCATATAGTATCACTCCCATCACAGCATTTCGATATCTTCAACCACTTTA ACCTTCTCCAGAGGATCATCATCTCAACACCGTCAAAATGGGAGACC	*P.chrysogenum* promoter sequence Pc24g00380 antifungal protein precursor paf
DsRed	GGTCTCGaatgGCCTCCAGCGAAGATGTCATCAAGGAGTTCATGCGCTTCAAG GTCCGCATGGAAGGATCCGTCAACGGCCACGAGTTCGAGATTGAGGGTGAGGGT GAGGGCCGCCCCTACGAAGGCACCCAGACTGCCAAGCTCAAGGTCACCAAGG GTGGTCCTCTCCCCTTCGCTTGGGATATCCTGTCTCCTCAGTTCCAGTACGGCTC CAAGGTCTACGTCAAGCACCCCGCCGACATCCCCGACTACAAGAAGCTTTCTTTC CCCGAGGGTTTCAAGTGGGAGCGTGTCATGAACTTCGAGGATGGTGGTGTTGT GACCGTTACTCAGGACAGCAGCTTGCAGGATGGCTCTTTCATCTACAAGGT CAAGTTCATTGGTGTCAACTTCCCCTCCGACGGCCCTGTCATGCAGAAGAAGAC CATGGGCTGGGAAGCGTCGACTGAGCGTCTGTACCCCCGTGACGGTGTTCT CAAGGGTGAGATCCACAAGGCTCTCAAGCTCAAGGACGGTGGTCACTACCTTGTT GAGTTCAAGTCCATCTACATGGCCAAGAAGCCTGTGCAGCTGCCCGGATACTAC TACGTGGACTCCAAGCTTGACATCACCTCCCACAACGAAGACTACACCATTGTT GAGCAGTACGAGCGTGCTGAGGGCCGCCACCACCTCTTCCTGACCCACGGAATG GATGAGCTGTACAAGTCGAAACTAtaaaGGAGACC	RFP (Red Fluorescent Protein) from *Discosoma* species with a 12 aa 'SKL' tag for peroxisomal targeting. Codon pair optimized for expression in filamentous fungi
Anid_ AN7354.ter	GGTCTCGtaaaTAAATGGTTTGCGTTGCGATTGACTGAAACGAAAAAAAGCGAAAAT GATTCTGGGAATGAATTGATAAAGCGCGGGCTCTGCGGTACGGTTACGGTTGCG GTCGCGGACGAATGGACTGGGCTGAGCTGGGCTGGAGGAAGTCCATCGAACAAG GACAAGGGGTGGAATATGGCACGGGTCGATTTTGTTATACATACCCTACCATC CATCTATCCATTTAAATACCAAATGAGTTGTTGAATGGATTCGCGGTCTTCTCG GTTTATTTTTGCTTGCTTGCGTGCTTAAGGGATAGTGTGcctcGGAGACC	Used as terminator for DsRed (*A.nidulans* terminator from possible ribosomal proteins L32)

Table S2. Oligonucleotide sequences used in this study

Oligo	Primer sequence (5'->3')	Purpose
5-5 IGR	AAGCGACTTCCAATCGCTTTGCATATCCAGTACCACAC-CCACAGGCGTTTCTAGGCTAAGGTCCGTTATC	Forward for 5' IGR cassette
a-5 IGR	AAAGCAAAGGAAGGAGAGAACAGAGGAGTACTTGTAC-GTTCGATGGGCAAGACTAAATCGGCTACTAGGC	Reverse for 5' IGR cassette
d-3 IGR	AACGTTGTCCAGGTTTGTATCCACGTGTGTCCGTTCCG-CCAATATTCCGCGATAGGTCTTCGGAGATAGAAG	Forward for 3' IGR cassette
3-3 IGR	ACTTAGTATGGTCTGTTGGAAAGGATTGTGGCTTCG-CATACAGGCTTTCTGATTCTCGTCGGAAGTACG	Reverse for 3' IGR cassette
b-Amds	CGGATCGATGTACACAACCGACTGCACCCAAACGAACA-CAAATCTTAGCAAAGCAGGCTCCTGGATCC	Forward for *amdS* cassette
c-Amds	CAACAGGAGGCGGATGGATATACTGTGGTCTGGAAGAT-GCCGGAAAGCGTGTACCGCTCGTACCATGG	Reverse for *amdS* cassette
A	ACCTAGGCTAAGGTCCGTTATC	Forward overlap GFP fragment
B	AGGGCATCAAGCTCACTAAC	Reverse overlap GFP fragment
C	TCCGCCTCTTCACCAAATCC	Forward overlap DsRed.SKL fragment
D	GATTCTCGTCGGAAGTACGGC	Reverse overlap DsRed.SKL fragment
5 IGR fw	GGATCCGGTCGCTAATATCG	Forward integration site check, overlap GFP fragment
GFP rv	GGTGTCACCCTCGAACTTG	Reverse integration site check, overlap GFP fragment
DsRed.SKL fw	GCTTCAAGGTCCGCATGGAAGG	Forward integration site check, overlap DsRed.SKL fragment
3 IGR rv	CTACCTCGTGGGATAGTCAG	Reverse integration site check, overlap DsRed.SKL fragment
γ actin gDNA fw	TTCTTGGCCTCGAGTCTGGCGG	Forward for copy number
γ actin gDNA rv	GTGATCTCCTTCTGCATACGGTCG	Reverse for copy number
eGFP fw	CCACCTACGGCAAGTTGAC	Forward for copy number
eGFP rv	GGTGTCACCCTCGAACTTG	Reverse for copy number
Ds Red-Skl fw	ATAAAGGCGGCTTCATGACC	Forward for copy number
Ds Red-Skl rv	AGTCTGGGTGCCTTCGTAG	Reverse for copy number
NiaD fw	TGATGGCTCCTCCAGGATG	Forward for copy number
NiaD rv	CGGGTGGATGGAAAGAGTC	Reverse for copy number